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Rupture of Base Pairing in Double-Stranded Poly(riboadenylic acid)·Poly(ribouridylic acid) by Formaldehyde: Medium Chain Lengths†

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ABSTRACT: By assuming that the opening of hydrogen bonds due to thermal fluctuations is a very fast step and that the reaction of formaldehyde with the imino or amino group is a slow step, we have constructed a model for the unwinding process of poly(A·U) induced by formaldehyde. The denaturation equation derived from the model is essentially the same as that of the zipper model for moderately long chain lengths. The model predicts the following phenomena which are in agreement with our experimental findings. The rate of unwinding is approximately first order for unfractionated polynucleotides and zero order for fractionated samples. This means that formaldehyde ruptures helical residues sequentially starting from the ends and working toward the center. Our model further predicts that the denaturation rate is linearly dependent on $-\log [Na^+]$ and on pH at low ionic strength and is almost independent of $[Na^+]$ and pH at high ionic strength. Spectrophotometric measurements on poly(A·U) were done to confirm our theoretical findings.

We undertook this study of the formaldehyde reaction with nucleic acids to obtain information on the unwinding behavior as well as on the secondary structure of nucleic acids in solution. We used double-helical poly(A·U) because it contains a single type of base pair but is still a good structural analogue of DNA.

In the past, Trifonov et al. (1967 and 1968), von Hippel and Wong (1971), and Doty and co-workers (Haselkorn and Doty, 1961; Utiyama and Doty, 1971) have also used formaldehyde as a probe for determining the secondary structure of nucleic acids and understanding the mechanism of unwinding. The formalization of bases in duplex DNA molecules will not occur unless the base pairs are opened. Therefore, the kinetic studies of the reaction will be useful for the quantitative investigation of defects in double-helical DNA such as single-stranded ends in λ-DNA, interruptions in polynucleotide chains in bacteriophage T5 DNA, and the hairpin structure in poly[d(A-T)] (von Hippel and Wong, 1971).

Under physiological conditions, DNA exists predominantly in the native double-helical form. Under such a mild condition, when the DNA molecule is essentially native, it is believed that thermal movement gives rise to some locally denatured regions. If the solution contains an agent that reacts with such unpaired nucleotides so that they can no longer form base pairs, unwinding will occur. Thus, the initial opening of a long DNA is due to the number of base pairs "breathing" cooperatively as a unit in native DNA. For this reason, the formaldehyde reaction has been used to find the "breathing units" in native DNA at temperatures well below the melting temperature (Frank-Kamenetskii and Lazurkin, 1974; von Hippel and Wong, 1971). Although various models have been proposed in the past, a rigorous statistical mechanical approach has not been carried out for the formaldehyde reaction. Trifonov et al. (1967 and 1968) have formulated an expression to estimate the fraction of base pairs at time t. Their expression, however, is not applicable to most experimental conditions, since an irreversible process was assumed in their derivation. In this paper, we approach this problem from the molecular level and derive a dynamic expression for the fraction of base pairs, which is applicable to moderately long polynucleotides. We confirm our theoretical predictions by carrying out experiments on poly(A·U). The denaturation works on very long polynucleotides will be reported elsewhere (Chay, 1976, 1977a,b).

Materials and Methods

The method of preparing formaldehyde stock solutions and measuring concentration has been described (Stevens and Rosenfeld, 1966). The polynucleotides were obtained from Miles Laboratories, Inc., and were purified by the phenol-bentonite procedure of Fraenkel-Conrat for RNA of tobacco mosaic virus (Fraenkel-Conrat and Singer, 1961). The poly(A) was lot number 16746 and had a s_{20} of 10.36; the poly(U) was lot number 41855 and had a s_{20} of 5.37. Concentration of the polymers was measured by UV absorption, the monomolar absorption coefficients at the respective absorption maxima

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TABLE I: Results of a Typical Fractional Precipitation of Poly(A) with NaCl.[a]

Fraction no.	NaCl (M)	Poly(A)[b] mg/mL	mg recovered	s_{20}[c]
1	0.53	1.30		
2, 3	1.07	1.07	21	12.2
4	1.18	0.56	119	10.3
5	1.24	0.41	30	8.4
6	1.30	0.24	30	7.0
7	1.94	0.04	53	4.8
8			13	3.4

[a] The NaCl concentration was measured with an Abbé refractometer before collecting the precipitate and the polymer concentration was measured afterward. [b] This is the concentration of poly(A) after filtration. Absorbance was measured in 0.2 M phosphate buffer, pH 7.4, at room temperature. [c] Measured at a concentration of about 10^{-4} M in 0.5 M KCl, 0.0067 M phosphate buffer, pH 7.4, at 20 °C. Values are uncorrected for density and viscosity.

being 10.4×10^3 for poly(A) and 9.9×10^3 for poly(U), both in 0.1 ionic strength solution (Stevens and Felsenfeld, 1964) at pH 7.0.

The poly(A) was fractionated according to molecular weight by fractional precipitation with NaCl. To begin, it was dialyzed against 0.5 M NaCl, 0.01 μ phosphate buffer, pH 6.8. We started with 250 mL of this preparation (1.33 mg of poly(A)/mL). Both the polymer solution and a saturated solution of NaCl were filtered through 0.45-μm Millipore filters under suction. The polymer solution was placed in a flask clamped to a water bath set at 25 °C. The saturated solution was added slowly until a noticeable turbidity was produced. The preparation was chilled in an ice bath until the turbidity disappeared and then warmed in the water bath. Upon reequilibration at 25 °C, the turbidity returned. The equivalent NaCl concentration was determined by measuring the refractive index with an Abbé refractometer. The polymer phase was separated by filtration through 1.2-μm Millipore filters. (If the solution sat too long before filtering, the precipitate tended to coalesce into water-clear droplets which stuck to the side of the vessel or settled to the bottom.) The filters with the isolated polymer fraction were placed in a vial and stored in the cold; later the polymer was taken up in 1–5 mL of distilled water and dialyzed into the desired buffer. This cycle was repeated for each fraction. The final filtrate was dialyzed against distilled water and the polymer was precipitated with 2 volumes of ethanol and a small amount of added NaCl solution. This was designated as the last fraction, but was not used in any experiments. Results of a typical fractionation are given in Table I.

Poly(U) did not precipitate with NaCl, so ethanol was added. The procedure was the same as for poly(A) except that the polymer, usually about 100 mg, was dialyzed into buffer. As a preliminary step, the solution at 25 °C was filtered through a 1.2-μm Millipore filter; any material retained by the filter was discarded. Absolute ethanol was added until turbidity developed. The precipitate was dissolved by heating to 60 °C and reprecipitated by cooling to 25 °C. Fractions were collected and removed from filters in the same way as in the fractionation of poly(A). Our samples of poly(U) behaved somewhat differently than poly(A); nearly all the polymer was present in the first fraction, indicating a rather narrow molecular weight range. On a typical preparation, we obtained 42 mg in the first fraction and 8 in the second.

The apparent weight-average molecular weight of samples used in studying the effect of pH on the kinetics, that is, of fractionated poly(A), poly(U), and poly(A·U), was determined by sedimentation equilibrium using the Rayleigh interference method with the Beckman Model E ultracentrifuge.

The poly(A) fraction had a sedimentation coefficient $s_{20} = 9.03 \pm 0.31$ and a degree of polymerization $Z_A = 771$ ($M = 268\,000$) (computed by the method of Inners and Felsenfeld, 1970). The poly(U) fraction had a sedimentation coefficient $s_{20} = 7.97 \pm 0.17$ and a degree of polymerization $Z_U = 867$ ($M = 279\,000$). The poly(A·U) sample was obtained by mixing in equal amounts poly(A) with poly(U) solutions of the same concentration, the mixture being then incubated for 30 min at 55 °C.

All equilibrium studies were carried at 20 °C and a light wavelength of 5460 Å was used. For poly(A) and poly(U) runs, the rotor speed was 6400 rpm and 3000 rpm for poly(A·U) runs. The loading concentration of the nucleotides was 0.5 mg/mL. The solvent in the poly(A) and poly(U) runs was 0.01 M phosphate buffer with 1.0 M NaCl added and a pH of 7.0. For poly(A·U) runs, the amount of added salt to the phosphate buffer was 0.05 and 0.1 M.

The data were analyzed using the method described by Van Holde and Baldwin (1958). The values of partial volumes and specific refractive increments were taken for poly(A), poly(U), and poly(A·U) from Eisenberg and Felsenfeld (1967), Inners and Felsenfeld (1970), and Cohen and Eisenberg (1968), respectively, with the corrections given by Casassa and Eisenberg (1964) due to different salt concentrations of the solvent.

We obtained for the apparent molecular weights: $M_A = 2.98 \times 10^5$ and $M_U = 2.82 \times 10^5$, which compare well with the values estimated from sedimentation coefficients. For the apparent molecular weight of poly(A·U), we found 8.23×10^5 and 8.58×10^5 for the two cases studied where the solvent was 0.01 M phosphate buffer + 0.05 M NaCl and 0.01 M phosphate buffer + 0.1 M NaCl, respectively. These values are approximately 1.4 times the sum of the apparent molecular weights of poly(A) and poly(U).

The kinetics of the formaldehyde reaction were determined with a Cary Model 14 spectrophotometer. All kinetic measurements were done at 35 °C in the presence of 1% (w/v) and 2% formaldehyde (0.33 and 0.67 M). Buffers were either 0.08 μ phosphate buffer, pH 7.4, or various concentrations of NaCl in 0.001 M phosphate buffer, pH 7.0 (measured after formaldehyde addition).

The stoichiometric coefficients for the complexes of poly(A) and poly(U) formed in 0.08 μ phosphate buffer and 35 °C were determined by the method of continuous variations (Stevens and Felsenfeld, 1964). These experiments show that the two-stranded complex, poly(A·U), forms and not the three-stranded one, poly(A·2U), under the conditions of these experiments. Moreover, the same experiments show that with unfractionated polymers formation of the A·U nucleotide pairs is not always complete. The hypochromism of 1:1 mixtures seems to be variable and depends on salt concentration, but typically we observe only about 80% of the maximum value obtained with high-molecular-weight polymers in the presence of 10^{-3} M $MgCl_2$. The hypochromism of the high-molecular-weight fractions in the ordinary buffers, however, is very near the maximum value. At the other extreme, the low-molecular-weight fractions under the same conditions gave only about 22% of the maximum value.

The combination of single-stranded poly(A) with single-stranded poly(U) to form the double-helical complex has an isosbestic point in the vicinity of 280 nm (Stevens and Felsenfeld, 1964). The addition of formaldehyde to poly(A) and to poly(U) results in isosbestic points at about 253 nm (Stevens and Rosenfeld, 1966) and about 267 nm, respectively (Eyring

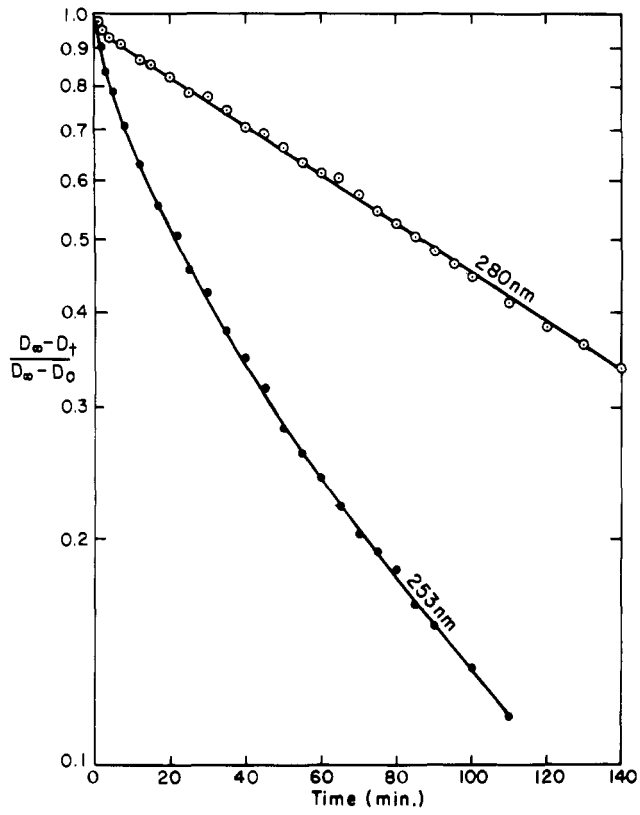


FIGURE 1: Kinetics of the reaction of formaldehyde with unfractionated poly(A·U). Spectrophotometric absorption was measured at 280 and 253 nm. The ordinate is defined in the text and gives the fraction of the reaction yet to occur at time t. Unless specified otherwise, all kinetic experiments were carried out with 0.08 μ phosphate buffer, pH 7.4, and 35 °C in the presence of 1% formaldehyde (w/v). The polynucleotide concentration was between 0.5 and 1.0 × 10^{-4} M in the residues.

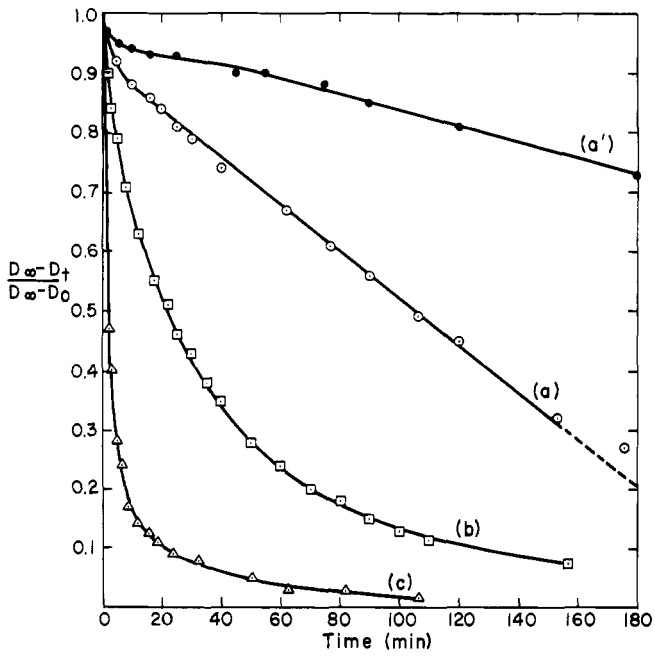


FIGURE 2: Kinetics of the reaction of formaldehyde with fractionated poly(A·U). (a) The poly(A·U) was prepared from high-molecular-weight fractions of the homopolymers and measured at 253 nm. (a') The same except measurements were made at 280 nm. (b) The poly(A·U) was prepared from unfractionated homopolymers and measured at 253 nm. (c) The poly(A·U) was prepared from low-molecular-weight fractions of the homopolymers and measured at 253 nm. (In 0.08 μ buffer the sodium ion concentration is 0.04 M.) The sedimentation coefficients of the homopolymer fractions were measured in 0.5 M NaCl, 0.01 μ phosphate buffer, pH 7.2, and 20 °C. The uncorrected values are poly(A), 9.9 and 5.5 S; poly(U), 4.6 and 1.8 S.

and Ofengand, 1967). Thus, when monitoring the reaction of formaldehyde with poly(A·U), at one of these that correspond to an isosbestic point, from the three reactions, base dissociation and reaction of formaldehyde with either A or U, only two of the three separate reactions are observable. At 253 nm, the rupture of nucleotide pairs and the addition of formaldehyde to free U residues are observed. These reactions occur as a single step, and the hyperchromicity is the sum of those for the separate reactions. At this wavelength, the hyperchromicity for the rupture of A·U pairs is about 36%, but only about −5% for formaldehyde addition to poly(U) (in 1% formaldehyde at 35 °C). At 280 nm, on the other hand, hyperchromicity for the addition of formaldehyde to A is about 70%, while that for addition to U is about +7%. At this wavelength, then, there is a small contribution from U which is of a different kinetic species. Except for these small contributions due to the formalization of free U residues, we can study independently the dissociation of the base pairs at 253 nm and the formalization of the free A residues at 280 nm.[1]

As shown by Haselkorn and Doty (1961) and by Stevens and Rosenfeld (1966), there is little likelihood that formaldehyde could be forming significant amounts of covalent cross-links (Feldman, 1962a,b) under the condition of our experiments.

[1] Haselkorn and Doty (1961) came to similar conclusions by analyzing the difference spectra of poly(A·U) in the helix and coil forms and in the presence and absence of formaldehyde. However, they found that the denaturation reaction could be observed at about 260 nm instead of 253 nm as we have reported. Their method involves comparison of spectra taken at 70 and 25 °C. Since most of the molecular species possess temperature-dependent hypochromism, such data may not be strictly applicable to reactions occurring at constant temperature.

Results

A typical example of the spectrophotometric kinetics of unfractionated poly(A·U) in formaldehyde appears in Figure 1. The spectrophotometric parameter, $(D_\infty - D_t)/(D_\infty - D_0)$, is plotted on the ordinate of the semilogarithmic plot. D_t is the absorbance at time t, and D_∞ and D_0 are the corresponding values in the limit of very long times and at zero time, respectively. This parameter gives the fraction of the total change in absorbance yet to occur; for one-step reactions, it gives also the fraction of reactant yet to be converted to products. Data were obtained at both 253 and 280 nm, representing, respectively, the rupture of hydrogen-bonded base pairs of the poly(A·U) and the addition of formaldehyde to the free amino groups of the A residues (see Materials and Methods). Since the kinetics depend quite markedly on the wavelength of measurement, it can be concluded that the reaction does not occur in a single step. Referring to Figure 1, one can see that the reaction of amino groups is very nearly first order, while the rupture of base pairs is of complex order. Also, the denaturation is faster than the formalization of free A residues.

The results of experiments with polymers which have been fractionated according to degree of polymerization appear in Figure 2. Here the spectrophotometric parameter, $(D_\infty - D_t)/(D_\infty - D_0)$, has been plotted on a linear scale against time. Curves a and a' result from the large molecular weight fractions at 253 and 280 nm, respectively. For these fractions the nucleotide pairing is nearly complete initially, yet the wavelength dispersion persists, showing again that the reaction does not occur in a single step. Curve b represents data for the unfractionated materials (from Figure 1) and curve c represents the low-molecular-weight fractions, both measured at 253 nm. One sees that the kinetics are markedly dependent on molec-

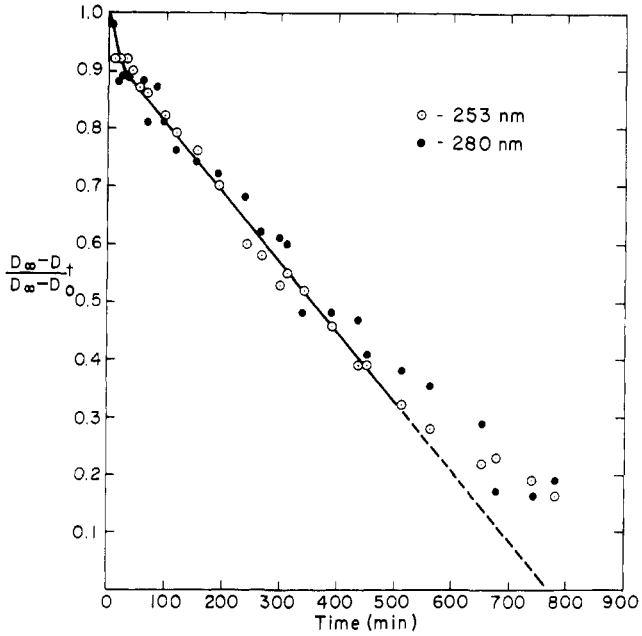


FIGURE 3: The conditions are the same as those for Figure 2a, except the solvent was 0.05 M NaCl, 0.001 M phosphate buffer, pH 7.0 (0.052 M in sodium ion). The homopolymers were high-molecular-weight fractions from another batch; the sedimentation coefficients were 10.3 and 5.9 S for poly(A) and poly(U), respectively. The open and closed symbols represent data taken at 253 and 280 nm, respectively.

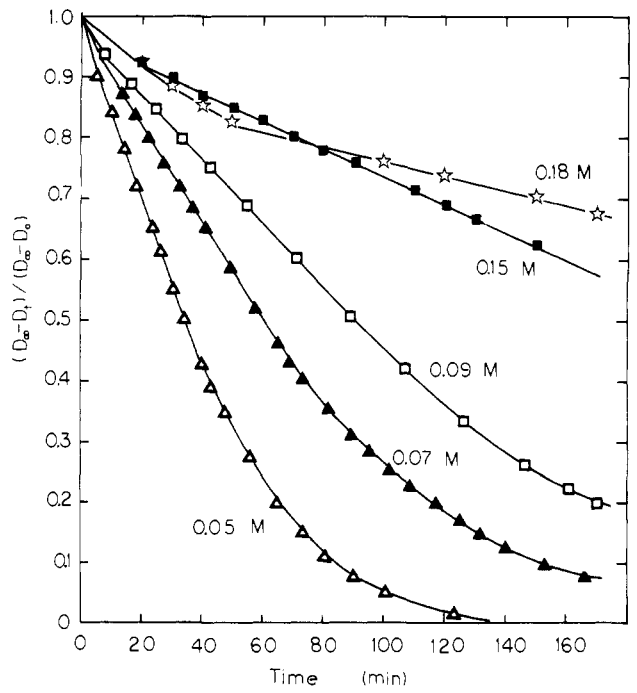


FIGURE 4: The dependence of the denaturation on Na^+ concentration. The solvent always contained 0.001 M phosphate buffer, pH 6.98, and 2% (w/v) HCHO with different salt concentrations added. The temperature was 35 °C. The degree of polymerization was computed by the method of Inners and Felsenfeld (1970) and was 342 for poly(A) and 363 for poly(U).

ular weight. For the low-molecular-weight fractions, the rupture of base pairs is virtually complete in 20 min, while only about 15% of the large molecules have ruptured in that time. (In the Discussion section, we will study the effect of molecular lengths on the unwinding.) What is perhaps more significant is the observation that the kinetics for the rupture of base pairs are of zero order over a substantial portion of the reaction (curve a). This leads to the hypothesis that formaldehyde attacks the end base pair of the double-helical complex resulting in its rupture and continues sequentially until all the base pairs have ruptured. One might expect that sooner or later the formaldehyde would be able to initiate reaction sites in the interior of helical regions of the molecules. The existence of zero-order kinetics shows that this does not happen to a significant extent under the conditions of our experiments. However, this situation is discussed further below.

The kinetics for samples with higher salt concentration are illustrated in Figure 3. The solvent was 0.05 M NaCl and 0.001 M phosphate buffer, pH 7.0. This gives a sodium ion concentration of about 0.052, compared to about 0.04 for the previous figure. The polymers used were high-molecular-weight fractions, but from a different batch; other conditions were the same. The uncorrected sedimentation coefficient of this fraction of poly(U) (in 0.5 M KCl, 0.006 M phosphate buffer, pH 7.5, at 20 °C) was 5.9 S. We corrected this figure to the viscosity and density of 1 M NaCl at 20 °C and computed the degree of polymerization from the empirical relationship given by Inners and Felsenfeld (1970). The result, 220, can be regarded as a minimum value. The degree of polymerization of the poly(A) fraction used was not estimated but is somewhat larger than that for the poly(U).

The zero-order kinetics are still observed over a large fraction of the reaction, but the dispersion of the denaturation and formalization process disappeared. This means that the criterion for a one-step reaction is now fulfilled and both reactions occur simultaneously.

Figure 4 shows the dependence of denaturation on Na^+ concentration. The solvent was 0.001 M phosphate buffer, pH 6.98, and 2% HCHO at 35 °C. The salt concentration was varied from 0.05 to 0.18 M. The double-helical poly(A·U) used in this experiment was prepared by mixing poly(A) (Z_A = 342) and poly(U) (Z_U = 363).

The dependence of the apparent zero-order rate constant for the denaturation of fractionated polynucleotides in the presence of 2% formaldehyde on added sodium chloride was determined from the central linear portion of the curves in Figure 4, and the result appears in Figure 5 (the points on the right-hand side of Figure 5). The points on the left-hand side of Figure 5 were obtained from the denaturation experiment in which 1% (w/v) formaldehyde was used instead of 2%, and the added sodium chloride varied from 0.2 to 0.09 M. The poly(A·U) were prepared from high-molecular-weight homopolymers. The central linear portion of the kinetic curves used in calculating the rate constant at 253 nm was not always as extensive as in curve a of Figure 2 and Figure 3, especially at the lower ionic strengths. For all curves, however, the central linear portion extends at least over 0.3 fractional density unit and is quite distinct from the initial transient and the final, nonlinear approach to equilibrium. At low ionic strength there is a rather large dependence of the rate on ionic strength. The rate becomes quite small, however, at higher ionic strength and appears to approach zero asymptotically. This effect will be discussed under Dependence of Denaturation on Ionic Strengths.

Figure 6 shows the effect of pH on the kinetics at low ionic strength (2% formaldehyde, 0.05 M NaCl in phosphate buffer). Here, the rates of denaturation and formalization increase as pH increases with the degree of polymerization Z = 771 for poly(A) and Z = 806 for poly(U) used. Figure 7 shows the effect of pH on the kinetics at high ionic strength. As seen in this figure, the denaturation is almost independent of pH. The conditions used were the same as those for Figure 6 except that

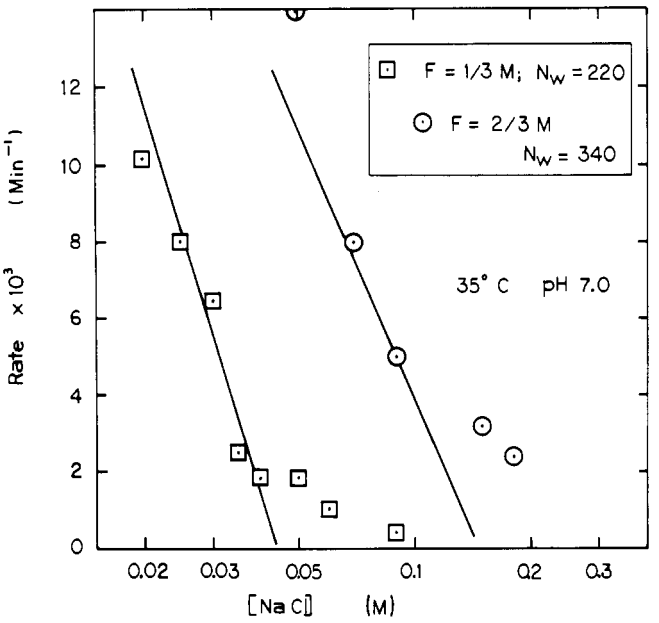


FIGURE 5: The dependence of the apparent zero-order rate constant on concentration of added salt in 1% HCHO (points on the left-hand side) and 2% HCHO (points on the right-hand side) solvents. For 2% HCHO, the rates were obtained from Figure 4. For 1% HCHO, the polymers were the same as described in Figure 3. The straight line in this figure was drawn from theory as described in the text.

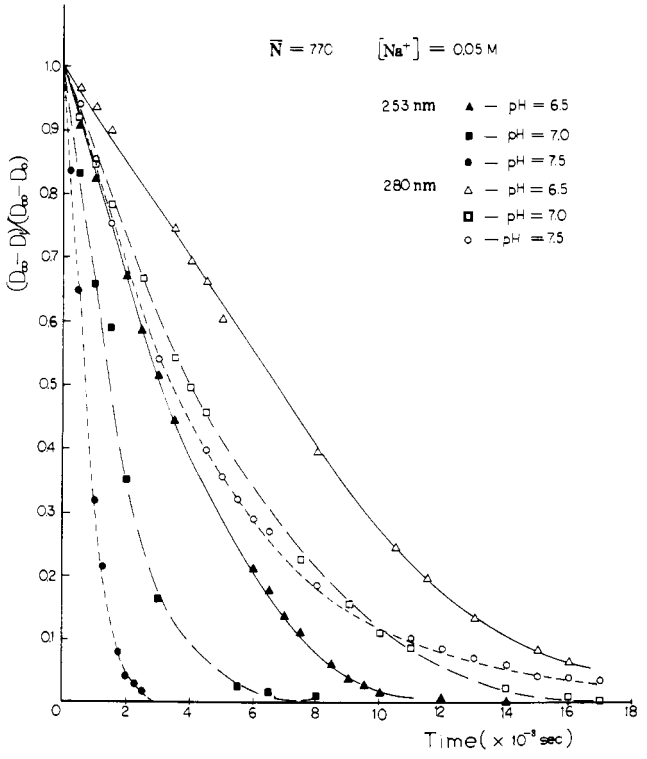


FIGURE 6: The dependence of pH on the kinetics at low ionic strength. The solvent contained 0.001 M phosphate buffer, 0.05 M NaCl, and 2% (w/v) HCHO. The temperature was 35 °C. The polymer samples used in the experiments have the degree of polymerization of 771 for poly(A) and 806 for poly(U).

the sodium concentration was increased to 0.09 M. Poly(A) used for the experiment has the degree of polymerization Z = 771 and poly(U) has Z = 719. This effect due to pH on the unwinding will be discussed under Apparent Reaction Rate vs. pH.

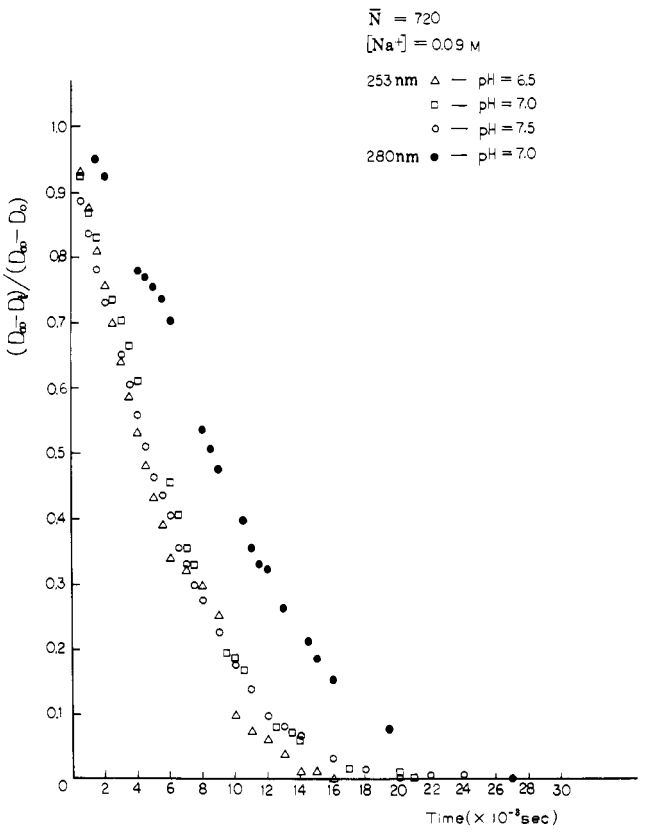


FIGURE 7: The dependence of pH on the kinetics at high ionic strength. The solvent contained 0.001 M phosphate buffer, 0.09 M NaCl, and 2% (w/v) HCHO. The temperature was 35 °C. Poly(A) has the degree of polymerization of 771 and poly(U) has 719.

Kinetic Theory of Unzippering and Formalization

The reaction of formaldehyde with synthetic polynucleotides and DNA has been studied by Haselkorn and Doty (1961), by von Hippel and Wong (1971), by Utiyama and Doty (1971), and by Trifonov et al. (1967, 1968; Lazurkin et al., 1970). A full theoretical interpretation of their experimental findings, however, has not been done yet, although different versions of the rupture mechanism have been postulated to account for the experimental findings. Among these, the rupture mechanisms proposed by Feldman (1973) and by von Hippel and Wong (1971) are most noteworthy: Feldman has postulated that the rupture of poly(A·U) is due to a complex process involving thermal denaturations and a fast formaldehyde addition to uridine residues. (This process of formalization to imino group cannot be observed spectrophotometrically, because HCHO is not tightly bound to the imino group at 35 °C.) Thus, formaldehyde added to the ring -NH-CO- group hinders renaturation and favors the less rapid formaldehyde reaction with the exocyclic amino group.

The mechanism proposed by von Hippel and Wong is that the base pair can be found in a denatured state due to thermal fluctuations and formaldehyde reacts only with the open form, i.e., unpaired bases. Thus,

$$\text{closed} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{open} + \text{formaldehyde} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \text{reacted} \qquad (1)$$

where k_1 and k_2 represent, respectively, the rate constant for the opening and closing reaction of the "breathing unit", k_3 the second-order rate constant for the reaction of formaldehyde with the open form, and k_4 the first-order rate constant for the back reaction representing the dissociation of the couples. In

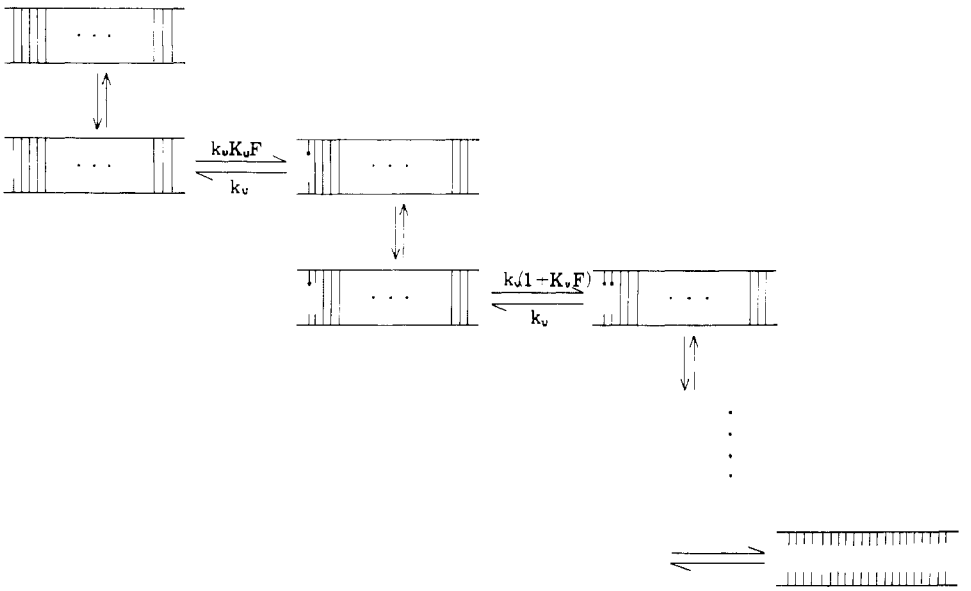


FIGURE 8: The denaturation process that is induced by formaldehyde. The processes along the vertical direction are due to thermal fluctuation, and the processes along the horizontal direction are due to the chemical reaction.

process 1, k_1 and k_2 are both very much larger than either k_3 or k_4, which means that the overall process can be viewed as a quasiequilibrium conformational transformation followed by much slower chemical step.

In this section, we derive a kinetic expression adopting the mechanisms proposed by von Hippel and Wong and by Feldman, which compare quantitatively with the experiments on poly(A·U).

Initially, all monomeric units (say N units) in poly(A·U) are completely paired except say the left-end one which is in thermal equilibrium with an open-end pair. We designate the completely paired species as poly(A·U)$_N$ and the open-end pair as poly(A·U)$_{N-1}$. Only the open-end base pairs react with formaldehyde, yielding the complex poly(A·U$_f$)$_{N-1}$. Because the breakdown and formation of paired bases occur much faster compared to the formalization process, the quasiequilibrium will be reached between the complexes poly(A·U$_f$)$_{N-1}$ and poly(A·U$_f$U)$_{N-2}$, before the latter reacts with formaldehyde. This reaction is shown by the reaction below (eq 2) (see also Figure 8). The ratio of the rates, k_2/k_1, is equal to s, the helix stability parameter of Zimm and Bragg (1959). The ratio k_3/k_4 is equal to K_U, the formalization equilibrium constant of the imino group.

$$\begin{array}{l} \text{poly(A·U)}_N \\ \quad \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \\ \text{poly(A·U)}_{N-1} + \text{HCHO} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \text{poly(A·U}_f\text{)}_{N-1} \\ \qquad\qquad\qquad\quad \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \\ \qquad\qquad\qquad\quad \text{poly(A·U}_f\text{U)}_{N-2} \end{array} \qquad (2)$$

This process of breaking and formalization in a sequential manner continues until the final step which frees the chains. This process is depicted in Figure 8. In this figure, the reactions along the vertical lines are known to be very fast (von Hippel and Wong, 1971), while those along the horizontal lines, which represent the reactions between HCHO and the imino group, are known to be much slower and therefore rate limiting (von Hippel and Wong, 1971).

Since the two species along the vertical lines (refer to Figure 8) are in quasiequilibrium with each other, by introducing the well-known rapid equilibrium assumption we find

$$C_0 \underset{k_b}{\overset{k_{f1}}{\rightleftharpoons}} C_1 \underset{k_b}{\overset{k_f}{\rightleftharpoons}} C_2 \underset{k_b}{\overset{k_f}{\rightleftharpoons}} \cdots \underset{k_b}{\overset{k_f}{\rightleftharpoons}} C_{N-2} \underset{k_{bN}}{\overset{k_f}{\rightleftharpoons}} C_{N-1} \qquad (3)$$

where

$$k_{f1} = \frac{k_U K_U F}{1+s}, \; k_f = \frac{k_U(1+K_U F)}{1+s}, \qquad k_b = \frac{k_U s}{1+s}, \; k_{bN} = \frac{k_U \sigma s}{1+\sigma s} \qquad (4)$$

(The derivation is given in Appendix A and also by Chay, 1976.) In the above equation, C_j is the concentration of poly(A·U) with the jth reacted site (i.e., the jth monomer bound to HCHO), s and σ are the helix stability and nucleation parameters of Zimm and Bragg (1959), respectively, F is the formaldehyde concentration, K_U is the binding constant of HCHO, and k_U is the rate parameter of HCHO addition to the imino group.

The helical content of poly(A·U) having N pairs at time t, $\theta_N(t)$, can be obtained from C_i by (see Appendix B for a detailed derivation and also Chay, 1976),

$$\theta_N(t) = \frac{1}{N}\left\{\sum_{i=0}^{N-2}(N-i)C_i - \frac{1}{1+s}[1 - C_{N-1}]\right\} \qquad (5)$$

The general form of kinetic equations resulting from eq 5 has been studied by Ree et al. (1962), by Pipkin and Gibbs (1966), by Chay and Stevens (1973), and Chay (1976). The time derivative of concentration C_i is, in general, a linear function of C_{i-1} (see Appendix B). If one makes use of this relation, eq 5 becomes (see Appendix B for a detailed derivation):

$$\theta_N(t) = \left[1 - \frac{(k_f - k_b)}{N}t\right] - \left[\frac{(k_b - k_f + k_{f1})}{N}\int_0^t \times C_0(t)dt + \frac{1}{N(1+s)}\int_0^t [1 - C_{N-1}(t)]dt\right] + \left[\frac{(k_f - k_b + \sigma k_{bN})}{N}\int_0^t C_{N-1}(t)dt\right] \qquad (6)$$

This kinetic equation consists of three terms. The first is a linear term inversely proportional to the number of segments N. The second is a transient; that is, its value becomes constant when $C_0(t)$ drops to zero which occurs early in the reaction if N is large. The third term is zero in the neighborhood of $t = 0$ and remains so until $C_{N-1}(t)$ becomes significantly greater than zero. The contribution from the third term does not come in for a substantial fraction of the total reaction time if N is large. Thus, for N sufficiently large and for $C_0(t) \simeq 0$ and $C_{N-1}(t) \simeq 0$, the model predicts a kinetic equation of the form

$$\theta_N(t) \simeq 1 - 2(k_f - k_b)t/N = 1 - \frac{2k_U}{N(1+s)}(1 + K_U F - s)t \quad (7)$$

where we have substituted $N/2$ for N in eq 6. This is because if we allow the reaction to proceed from both ends, but require that the reaction proceeds symmetrically and ends exactly in the middle, then N in eq 7 should be one-half the degree of polymerization. When N refers to the degree of polymerization, we insert $N/2$ in the equation. (Note that N hereafter is the same as Z under Materials and Methods.)

Equation 7 provides the theoretical framework for interpreting our experimental curves. Indeed, $\theta_N(t)$ represents the fraction of helical residues still present at time t and can be represented by the spectrophotometric parameter $(D_\infty - D_t)/(D_\infty - D_0)$ as measured at 253 nm. The three stages of the reaction are seen clearly in Figures 2 and 3. There are an initial transient, a central linear portion, and a gradual approach to equilibrium.

Dependence of Denaturation on Ionic Strength

Because the rate of formalization of free U residues is greater than the formalization of free A residues, the rate of rupture is equal to

$$\text{rate} = \frac{2k_U}{N(1+s)}(1 + K_U F - s) \quad (8)$$

where the subscript U refers to the reaction between HCHO and the imino group. It is desirable to see whether or not the model is in quantitative accord with the experimental results. One way to do this is to see if eq 8 gives the correct relationship between the apparent rate of reaction in the linear portion (Figure 5) and other parameters which are more or less accessible from experiment.

First, we wish to determine the functional form of eq 8 with respect to ionic strength but constant temperature. It has been observed that for many double-helical nucleic acids, T_m, the "melting temperature", is a linear function of log concentration of neutral, uni-uni valent salt in the medium (e.g., Doty et al., 1959). Stevens and Felsenfeld (1964) have found that T_m of poly(A·U) is related to log $[Na^+]$ by

$$\log [Na^+] = 0.054(T_m - 303.15) + \log 0.0045 \quad (9a)$$

Assuming that $\Delta H°$ of helix formation is independent of temperature (this is not true generally for polynucleotides, but it is usually a good first approximation), and applying the van't Hoff equation, one obtains

$$s = \exp[\Delta H°(T - T_m)/RT_mT] \quad (9b)$$

Equation 9b implies that there is a linear dependence of log s on T_m if $T_m - T$ is not too large. Thus, we expect a linear dependence of log s on log [NaCl] at a constant temperature T. By differentiating the right side of eq 8 with respect to s and multiplying by s, we find that the variation of the apparent rate, $2(k_f - k_b)/N$, with ln s is given by

$$\frac{\partial \text{ rate}}{\partial \ln s} = \frac{-2k_U}{N}\frac{s}{(1+s)^2}(2 + K_U F)$$

The parameters k_U and K_U are independent of salt concentration (Aylward, 1966). The factor $s/(1 + s)^2$ is a weak function of s; it has a maximum value of 0.22 when s is 2 and 0.5. This range includes the values of s ordinarily encountered. In the experiments for which eq 8 applies, this factor varies only by about 3%. Thus, if eq 8 is of the correct form, the apparent zero-order rate constant should vary approximately linearly with log salt concentration. The points on Figure 5 between 0.02 and 0.04 molar added sodium chloride (i.e., the points on the left-hand side, where the HCHO concentration used is 1%) and the points between 0.05 and 0.09 molar added sodium chloride (2% HCHO concentration) are described satisfactorily by a straight line. The points at higher ionic strength depart from this line, but it is clear that the formalization of U residues is not rate limiting in that region and eq 8 no longer applies.

Equation 8 gives a convenient method for obtaining the value of K_U, the binding constant of poly(U), and the value of k_U, the rate parameter. This can be done as follows: Using eq 9a for the dependence of T_m on salt concentration, the value of -6500 cal mol^{-1} for $\Delta H°$ obtained by Ross and Scruggs (1965), and eq 9b, we evaluate K_U by extrapolating the linear portion of Figure 5 to zero rate, since at this ionic strength $s = 1 + K_U F$. After obtaining K_U, we compute the value of k_U by fitting the linear portion of Figure 5 using eq 8. The value of N in eq 8 can be determined from sedimentation measurements.

We have found that the extrapolation yields [NaCl] = 0.046 M for 0.33 M HCHO (left-hand curve of Figure 5) and [NaCl] = 0.137 M for 0.667 M HCHO (right-hand curve). With these values and by using eq 9a and 9b, we have found $s = 1.57$ for 0.33 M HCHO and $s = 2.06$ for 0.667 M HCHO. From the relation $1 + K_U F = s$, we have obtained the value of K_U to be about 1.65 M^{-1}.

Aylward (1966) has presented equilibrium data obtained by several different methods for both UMP and poly(U) in formaldehyde at several temperatures and pH. Interpolating his data for UMP at 35 °C, K_U is about 1.7 for the spectrophotometric method and about 1.5 for the pH elevation method and pH-statting method. Data for poly(U) could not be obtained so easily, but he estimated values of K_U from the pH-statting method which, upon interpolation, gives a value of about 1.75. This value is within experimental error of that estimated from the data of McGhee and von Hippel (1975a,b). McGhee and von Hippel have reported that the equilibrium constant, K_U, for poly(U) is equal to 2.30 M^{-1} at 24 °C. The enthalpy change $\Delta H°$ for poly(U) has not been measured by these workers, but $\Delta H°$ for 5′-TMP has been reported as -3.4 ± 0.3 kcal. Assuming that $\Delta H°$ for poly(U) is about equal to that for 5′-TMP, we find K_U to be 1.87 M^{-1} at 35 °C. Thus, our value of K_U by extrapolating the data of Figure 5 to "zero rate" is in satisfactory agreement with the value obtained by Aylward and by McGhee and von Hippel in a direct way.

Equation 8 yields a straight line as shown in Figure 5 with s computed as described above and the following values, $K_U = 1.65$ M^{-1}, $k_U = 10$ min^{-1}, and $N = 220$ for $F = 0.333$ M and $N = 340$ for $F = 0.667$ M, where N values were obtained from sedimentation measurements and the relation of Inners and Felsenfeld (1970).

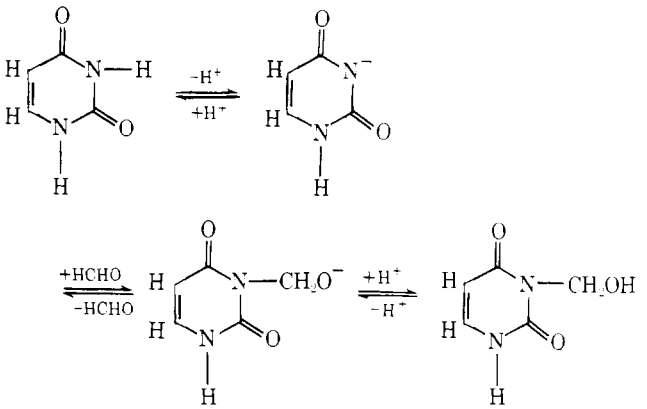
Again, from the data of McGhee and von Hippel (1975a,b), we can estimate the value of k_U. Experimental values of k_U for

5′-UMP and poly(U) have been reported by McGhee and von Hippel as 0.057 ± 0.003 and 0.011 ± 0.001 s^{-1}, respectively, at 25 ± 0.2 °C and pH 6.95. The enthalpies of activation and reaction for 5′-UMP and poly(U) have not been reported by these workers, but for 5′-TMP these values are $\Delta H^{\ddagger}$ = 25.1 ± 1.4 kcal and $\Delta H°$ = 3.4 kcal, respectively. Assuming that $\Delta H^{\ddagger}$ and $\Delta H°$ for 5′-UMP and poly(U) are equal to those of 5′-TMP, we obtain k_U for 5′-UMP to be 14.8 min^{-1} and k_U for poly(U) to be 3.15 min^{-1}. Our computed value k_U = 10 min^{-1} falls between these values. Thus, it seems that our experimental data for poly(A·U) are in fairly good quantitative accord with the model leading to eq 8, at least at low ionic strength for which formalization of U residues is rate limiting.

Apparent Reaction Rates vs. pH

Utiyama and Doty (1971) have proposed that the denaturation step observed at 253 nm at low ionic strength or high temperature is due to an induction effect of HCHO. This contradicts our hypothesis that the denaturation observed at 253 nm is mainly due to the reaction of HCHO with the imino group and that the reaction observed at 280 nm is that of HCHO with the amino group. In order to verify our hypothesis, we have studied the effect of pH on the denaturation. Earlier, McGhee and von Hippel (1975a,b) showed that the HCHO–imino reaction is base catalyzed in the pH range about 4 to 9, while the HCHO–amino reaction is not.

The effect shown in Figures 6 and 7 can be explained quantitatively as follows. In the derivation leading to eq 8 it was assumed that the pH of media is held constant. If the pH of media is not fixed, the apparent rate expression can be modified by using the HCHO–imino reaction mechanism proposed by McGhee and von Hippel (1975a,b). These workers have proposed that the reaction mechanism is an equilibrium deprotonation step, followed by attack of the negatively charged nitrogen on the carbon atom of the unhydrated formaldehyde molecule, approaching perpendicular to the plane of the carbonyl group. The final step would then be the fast reprotonation of the, now much more basic, ionized methylol group. Thus, the suggested mechanism can be diagrammed as follows (McGhee and von Hippel, 1975a,b):



If one takes this reaction mechanism into consideration and assumes that the protonation step is very fast compared to the formalization step, eq 8 becomes

$$\text{rate} \simeq \frac{2k_U\left(1+\frac{10^{-7}}{K_a}\right)}{N(1+s)\left(1+\frac{[H^+]}{K_a}\right)}(1+K_UF-s) \qquad (10)$$

where K_a is the acid-dissociation constant for imino group ($\simeq 10^{-10}$) and k_U is the rate parameter measured at pH 7. The stability of DNA is relatively insensitive to pH between 5 and 9 (Bloomfield et al., 1974), and hence we may assume that s in eq 10 is independent of pH. This means that for pH $\ll$ pK_a, eq 10 becomes

$$\text{rate} \simeq 10^{-7+\text{pH}}\frac{2k_U}{N(1+s)}(1+K_UF-s) \qquad (11)$$

or

$$\log \text{rate} \simeq \text{pH} + \text{constant}$$

Thus, at the pH range between 5 and 9, log rate vs. pH should yield a straight line with a slope unity. Figure 6 shows the effect of pH on the kinetics. The condition used for the experiment is well within the range where $1 + K_UF > s$ is satisfied. Thus, according to our model, the denaturation is due to the reaction between poly(U) and HCHO. The apparent rates found from Figure 6 do not quite give eq 11; i.e., the slope of log rate vs. pH is not quite unity. It is probably because the length of polymer used for the experiment (Z = 771) is so long that poly(A) may be also involved in the denaturation process.

At high salt concentration, the apparent rate should be insensitive to pH because, according to our model, the rate-determining step is due, in this case, to the HCHO–amino reaction which is not a base-catalyzed reaction. The condition used for the experiment yields $1 + K_UF - s \simeq 0.2$, which indicates that the condition of the experiment lies on the border line where the rate-determining step switches from U to A. As shown in Figure 7, the denaturation is almost independent of pH.

Reaction with Denatured Amino Groups of A (i.e., Reaction Observed at 280 nm)

Since the amino group of poly(A) reacts much slower than the imino group, the reaction of the amino group with HCHO should trail the denaturation. This has been observed in the experiment shown in Figure 2 (i.e., the reaction rates at 253 and 280 nm are different). In this section we derive the expression for the formalization of A.

The simple reaction with single-stranded poly(A) was treated by Stevens and Rosenfeld (1966). It was shown that

$$\frac{dA_f}{dt} = k_AK_AFA_d - k_AA_f \qquad (12)$$

where A_d and A_f are the concentrations of unreacted and reacted A residues, respectively. Here k_A is the reverse first-order rate constant and K_A is the binding constant of A. The apparent pseudo-first-order rate constant is

$$k_a' = k_A(1 + K_AF) \qquad (13)$$

Relation 13 can be used here with the provision that A_d and A_f refer to the free and the formalized A residue, respectively, at any time t. The conservation equation becomes

$$A_0 = A_n + A_f + A_d \qquad (14)$$

where A_0 is the total concentration of A residues, and A_n is the concentration of hydrogen bonded ones at time t. The quantity A_n should then be equal to

$$A_n = A_0\theta$$

where θ is the helical content. By substituting this expression into eq 14, we find

$$A_d = A_0(1-\theta) - A_f$$

Then, the differential eq 12 becomes

$$\frac{dA_f}{dt} = k_A K_A F A_0 (1 - \theta) - k_a' A_f$$

or

$$\frac{A_f}{A_0} = e^{-k_a' t} \int_0^t k_A K_A F (1 - \theta) e^{k_a' t} dt \qquad (15)$$

At equilibrium, we have $A_{f\infty}/A_0 = K_A F/(1 + K_A F)$. Thus, the fraction of A residues yet to react at time t is given by

$$Y_A(t) = 1 - \frac{A_f/A_0}{K_A F/(1 + K_A F)} \qquad (16)$$

which is an experimentally observable quantity and can be obtained numerically by using $\theta(t)$ given by eq 6.

An approximate form of $Y_A(t)$ can be obtained from eq 15, since $\theta(t)$ takes the form $\theta(t) \simeq 1 - 2(k_f - k_b)t/N$ for a large fraction of the reaction. By substituting this form into eq 15 and integrating, we find

$$Y_A(t) \simeq 1 - \frac{2(k_f - k_b)}{N} [t - (1 - e^{-k_a' t})/k_a']$$

or

$$Y_A(t) - \theta(t) = \frac{2(k_f - k_b)}{N k_a'} (1 - e^{-k_a' t})$$

$$= \frac{2k_U(K_U F + 1 - s)}{N k_A (1 + s)(1 + K_A F)} (1 - e^{-k_a' t}) \qquad (17)$$

As we have indicated before, the kinetics measured at 280 nm will represent mainly the formalization of free A residues given by $Y_A(t)$. The denaturation process is measured at 253 nm and is represented by $\theta(t)$. The left-hand term is what Utiyama and Doty (1971) referred to as the unreacted fraction. Equation 17 says that, even under the most favorable of conditions, the reaction of A residues with formaldehyde is slower than the rupture of base pairs by the factor of $2(k_f - k_b)(1 - e^{-k_a' t})/N k_a'$. When this term is much greater than unity, the base pairs are ruptured very early in the reaction. The formalization of poly(A) proceeds almost as if the poly(U) were not there, and the kinetics is of first order (observed at 280 nm). Such a behavior is expected with short polymers or in low salt, as shown in Figure 2. When $k_f \simeq k_b$ the second term in eq 17 becomes close to zero, and the formalization of poly(A) and poly(U) occurs simultaneously. Both reactions are zero order, and the dispersion of the spectrophotomeric parameter disappears. These phenomena are observed in the experiments on poly(A·U) as shown in Figure 3.

In Figure 9 we have compared the experimental denaturation and formalization data with the theory for $[Na^+] = 0.09$ M, [HCHO] = 0.667 M. The theoretical value has been computed by using eq 6, 16, and 19 and the analytical expression for C_0 and C_N given by Chay and Stevens (1972). The parameters used in this figure are $k_A = 0.0018$ min^{-1} and $K_A = 10$ M^{-1} which came from the experimental measurements of Stevens and Rosenfeld (1966) and $k_U = 10$ min^{-1} and $K_U = 1.65$ M^{-1} which were obtained from Figure 5. In this figure, χ_r and χ_d are the reacted and denatured fractions, respectively, and are defined as

$$\chi_r = 1 - Y_A, \chi_d = 1 - \theta - \chi_r \qquad (18)$$

We find a fair agreement among all three quantities, θ, χ_d, χ_r, for this ionic strength.

When the ionic strength is high and/or the formaldehyde concentration is low, the formalization of A and denaturation will occur simultaneously. In the next section, we will formulate an expression for the denaturation rate when the rupture and the formalization of A occur simultaneously.

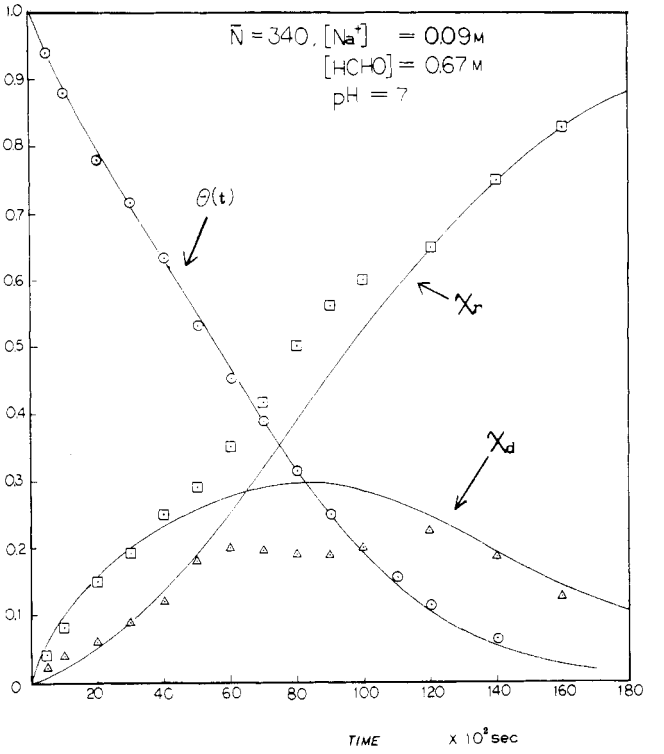


FIGURE 9: Variation with time of three quantities: the reacted fraction χ_r, the denatured but not yet reacted fraction χ_d, and the natured state θ. The circles are the experimental data taken from Figure 4. The solid lines were calculated from the theory as discussed under Reaction with Denatured Amino Groups of A of the text.

Denaturation Process at High Ionic Strength: Rupture Process Due to the Amino Group of Poly(A)

Experimental works on poly(A) and poly(U) show that the rate of reaction between HCHO and the imino group is different from that between the amino group by a factor of 10^4, the latter being the much slower step (McGhee and von Hippel, 1975a,b). Therefore, the formalization reactions of the imino and of amino groups occur in two well-separated stages. That is, at sufficiently high temperature and low ionic strength the reaction between HCHO and the free imino group is sufficient to rupture the helices, and hence the reaction of HCHO with the free amino group will trail the denaturation process. However, at high ionic strength and/or very low temperature, it is the reaction between HCHO and the amino group which ruptures the helices. Our model predicts that this switch occurs at $s - 1 \simeq K_U F$; i.e., the rate as given by eq 12 becomes about zero. When this condition is met, the rate-determining step is no longer due to the imino group, and hence the model should be revised.

Since the reaction of HCHO with the free imino group is very fast, the system is not only in rapid equilibrium with the thermally induced helix–coil transition but also with the transition induced by the reaction between the imino group and HCHO. Thus, the rapid equilibrium exists between a completely natured state and a thermally as well as chemically denatured state. The first denaturation step is the same as the scheme shown in eq 2, except that the ratio of the rates k_2/k_1 is no longer the helix stability parameter s but is equal to

$$k_2/k_1 = s/(1 + K_U F)$$

and the formalization rates k_3 and k_4 are, respectively, $k_3 = k_A K_A$ and $k_4 = k_A$, where K_A is the binding constant of poly(A). As in the case of the formalization process of imino

group (see Kinetic Theory of Unzippering and Formalization), the complete reaction is given by eq 4 with the following revised rate parameters:

$$k_f = \frac{k_A(1 + K_A F)(1 + K_U F)}{1 + K_U F + s}, \quad k_b = \frac{k_A s}{1 + K_U F + s}$$
$$k_{fN} = \frac{k_A K_A F(1 + K_U F)}{1 + K_U F + s}, \quad k_{bN} = \frac{k_A \sigma s}{1 + K_U F + \sigma s} \tag{19}$$

The apparent rate of rupture (i.e., the slope of the linear portion of the denaturation profile) will be then

$$\text{rate} \simeq \frac{2k_f}{N}[1 - s(f)] \tag{20}$$

where

$$s(f) = \frac{s}{(1 + K_U F)(1 + K_A F)} \tag{21}$$

Schellman (1958) and Bixon and Lifson (1966) have shown that eq 21 is the correct form for the statistical weight of free residue in equilibrium with a specific solvent component.

We have tried to fit eq 20 to the data of Figure 5 for 0.33 M HCHO at 0.05 M sodium chloride and above, using data obtained by Stevens and Rosenfeld (1966) for $k_A = 0.0018$ min^{-1} and $K_A = 10$ M^{-1}. The calculated denaturation rates are much smaller than the experimental data. For example, the calculated rate is equal to 2.3×10^{-5} min^{-1} at [Na$^+$] = 0.09 M, while the experimental value is 4.5×10^{-4} min^{-1} at this Na$^+$ concentration. A probable explanation for the failure of eq 20 is that in the derivation leading to eq 20 we have assumed that the formalization process is followed by the formation of one coil (i.e., the breakage of one pair of hydrogen bonds). At these ionic strengths and HCHO concentrations, however, the rapid equilibrium has been established by the chemical reaction (i.e., the reaction between imino group and HCHO) as well as by thermal fluctuations. Thus, it is reasonable to assume that each formalization process (of amino groups) induces the breakage of two or more pairs of hydrogen bonds.

Equation 20 says that the reaction rate will fall to zero when $s = (1 + K_U F)(1 + K_A F)$. The value of K_A at 35 °C is about 10 M^{-1} (Stevens and Rosenfeld, 1966). Using the values for the other parameters already given, one finds a value of about 6.9 for s if the reaction rate is to be zero. But it would take much more than 1 M sodium chloride to achieve such stability in poly(A·U), if indeed such stability could be achieved by changing ionic strength alone. This means that the rate constants in Figure 5 can be expected to approach zero almost asymptotically in the decade 0.1 to 1 M sodium chloride, and even higher. This explains why, under some conditions, nucleic acids can take an extremely long time to equilibrate with formaldehyde, especially if the temperature is far below that of the melting transition.

Discussion

In the areas in which they overlap, our experimental results are mainly in agreement with those reported earlier by Haselkorn and Doty (1961). While these workers used unfractionated polymers, our experiments depend critically upon the fractionation of poly(A) and poly(U) according to molecular weight prior to forming the complex. We do not find, however, that the denaturation reaction is first order for fractionated polymers and is approximately so for unfractionated polymers. A likely explanation for this lies in the sensitivity of the reaction to molecular weight, or more specifically to the size distribution of helical regions. Thus, the apparent order of the kinetics depends upon the dispersity of the preparations used, which probably differed between the two groups. Trifonov et al. (1968) have shown that if the lengths of helical sections are distributed in a random fashion then the denaturation reaction will be first order (assuming no new reaction sites). It can be also shown that if one averages $\theta(t)$ given by eq 15 over the probability functions of type

$$\Phi_N = \frac{1}{\overline{N}}\left(1 - \frac{1}{\overline{N}}\right)^{\overline{N}-1} \tag{22}$$

where $\overline{N}$ is the average number of hydrogen bonds (or $1/\overline{N}$ is the concentration of defects at $t = 0$), we find the degree of helicity decays exponentially with the expression (Chay, 1976, 1977a)

$$\langle\theta(t)\rangle \simeq \exp\left\{-\frac{2k_U}{\overline{N}(1 + s)}(1 + K_U F - s)t\right\} \tag{23}$$

The conditions in the experiments of Doty and Haselkorn were $F = 2.76/3$ M, [K$^+$] = 0.165 M, pH 6.8, at 35 °C. With the values $k_U = 10$ min^{-1}, $K_U = 1.65$ M^{-1}, and $s = 2.2$ (calculated from eq 9), we find from eq 23 the average chain length of poly(A·U) is $\overline{N} \simeq 44$. This value indicates that the samples used by Haselkorn and Doty were very low-weight fractions.

Equation 23 represents a random distribution of sizes or ends. Considering the enzymatic nature of the synthesis of polynucleotides, it is not unexpected that preparations might approach more or less closely a random distribution of sizes. For the smaller polymers, then, the apparent order of the kinetics is strongly influenced by their size. In this study, this factor has been largely eliminated by selecting the longest polymers. In our samples, however, even the longest polymers do not approach the size of natural DNA. In the later discussion and also in Chay (1977a,b), we will study in detail the effect of chain lengths on the unwinding of DNA and poly(A·U).

We do observe that at low ionic strength the formaldehyde reaction occurs in two steps, an initial denaturation step followed by the formalization of free A residues. From the work of Lewin (1962, 1964), Aylward (1966), and Eyring and Ofengand (1967) with UMP and poly(U) as well as that of Haselkorn and Doty (1961), Stevens and Rosenfeld (1966), and McGhee and von Hippel (1975a,b), it is clear that the initial denaturation step consists of a configurational transition (rupture of hydrogen-bonded base pairs) and the formalization of the acid imino group of U. The second slower step is the formalization of the amino group of A. We have proved our hypothesis that the denaturation is due to the HCHO–poly(U) reaction by showing the pH effect on the kinetics, as shown in Figures 6 and 7.

Haselkorn and Doty showed that, with poly(I·C) at high ionic strength, the reaction occurs in a single step. Figure 3 shows that this occurs with poly(A·U) also. An explanation for this has been presented under Denaturation Process at High Ionic Strength: Rupture Process Due to the Amino Group of Poly(A). The value of s is an increasing function of the ionic strength of the solvent, but K_U and K_A are largely independent of ionic strength. If $1 < s < 1 + K_U F$, the reaction is limited only by the comparatively fast formalization of U residues. The reaction with the A residues thus freed lags the denaturation reaction, and the overall reaction is observed to occur in two steps. On the other hand, if $1 + K_U F < s < (1 + K_U F)(1 + K_A F)$, the reaction is no longer limited only by U but seems to be in transition from U limited to A limited or is simply an A-limited reaction. As indicated by the spectra, however, the reaction occurs in a single step throughout this range. For $s > (1 + K_U F)(1 + K_A F)$, the formaldehyde-induced unwinding

is incomplete and some base pairs remain at equilibrium.

We do not agree unconditionally, as Haselkorn and Doty have stated, that the rate of addition of formaldehyde to A residues does not depend upon whether the amino groups are completely and strongly hydrogen bonded as in poly(A·U) or occasionally and weakly hydrogen bonded as in poly(A). (It is now known that, except below pH 6, poly(A) is not intermolecularly hydrogen bonded at all.) Their statement is largely true at low ionic strength where the A·U pairs are weakly bonded and the denaturation reaction is very much faster than the formalization of amino groups. At higher ionic strength, however, the A·U pairs are more strongly paired, and the rate of addition of formaldehyde to hydrogen bonded A residues is very much slower than to free residues. This can be seen from a comparison of Figures 2 and 3.

We have found that the complete denaturation or unzippering reaction of fractionated poly(A·U) occurs in three stages. The first is a transient making up about 10% of the complete reaction. It represents the stage for which a significant fraction of the polymers have not reacted with formaldehyde at all. The second stage, about 70% of the reaction, represents the sequential rupture of base pairs. This stage is interpretable in terms of a reversible sequence of reactions: the spontaneous unpairing of the end base pair and the subsequent formalization of the free acid imino group. The overall rate constant for this stage seems to be adequately described by the product of two probabilities, the probability that the end base pair is not bonded times the probability per unit time that a free base will be formalized. Depending upon the innate stability of the base pair, the amino group of A may or may not be formalized in this step. Because the half-time for the reaction with free acid imino groups is generally several orders of magnitude smaller than that for free amino groups and the respective equilibria are of the same order, the overall reaction is dominated by the former when the pairing is only weakly stabilized, the reaction being always significant.

The strength of pairing can be related to the parameter $T_m - T$, where T_m is the helix–coil transition temperature in the absence of formaldehyde, and T is ambient temperature. Our solutions contained between 0.02 and 0.1 M added sodium chloride for which T_m varies between about 42 and 56 °C (Stevens and Felsenfeld, 1964) and ($T_m - T$) between 7 and 21 °C. In the critical region between 0.04 and 0.05 M salt (Figure 5), ($T_m - T$) is about 15 °C in 0.33 M formaldehyde. It appears that the value of ($T_m - T$) appropriate to this critical region, however, depends upon the formaldehyde concentration, as shown on the right-hand side of Figure 5.

The third stage of the denaturation reaction comes about when some of the molecules no longer have any base-paired residues, and the system approaches equilibrium more or less exponentially. It is expected that the kinetics in this stage are sensitive to the degree of homogeneity in the degree of polymerization of the constituent polymers. Our experimental curves, however, are described quite well in this respect by the exact theory discussed under Kinetic Theory of Unzippering and Formalization (see Figure 8) for which a single degree of polymerization was assumed.

We wish to cite some current results on the formaldehyde reaction with DNA which have particular relevance here. Lazurkin and his colleagues (summarized in Lazurkin et al., 1970) showed that formaldehyde can react with native DNA starting at the ends of the paired chains or at other reaction centers caused by physical damage to helical base pairs, such as that from ionizing radiation. In addition, reaction centers exist within ordered helical regions of DNA. They arise presumably from the spontaneous and simultaneous unpairing of

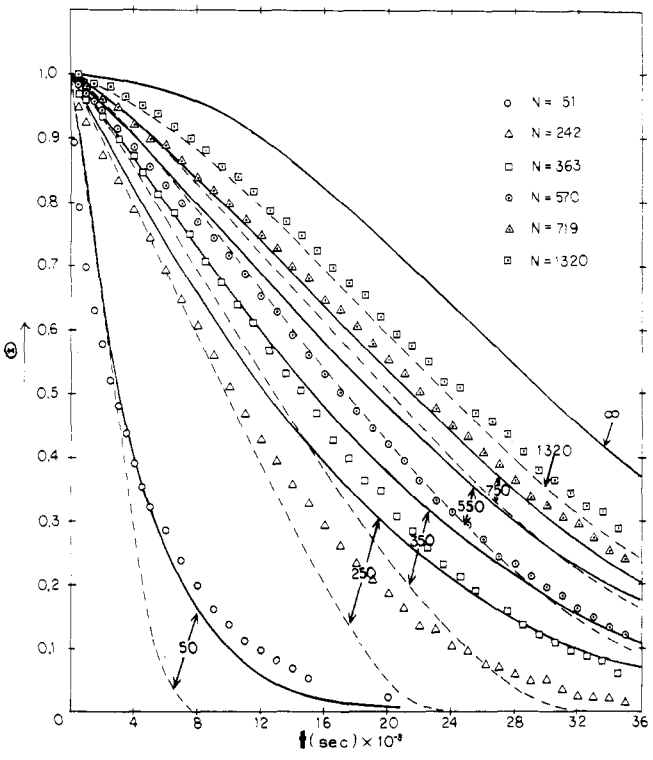


FIGURE 10: The conditions are the same as those for Figure 3. The theoretical curves (i.e., the dashed and solid lines) were obtained by using the master equation with the following parameters: $s(0) = 2.0$, $s(f) = 0.24$, $\sigma = 2 \times 10^{-5}$, and $k_b = 7.44 \times 10^{-3}$ s^{-1}.

several helical residues which allows them to react with formaldehyde and which in turn initiates a sequential rupture of base pairs. This approach to studying the "breathing" of helical DNA has also been utilized by von Hippel and Wong (1971). They found that about 9 base pairs were involved in a reaction center and that this number remains constant between 10 and 40 °C below the melting transition zone.

It is clear that with poly(A·U) under the conditions of our experiments, we observe only the reaction from the ends; there are no detectable numbers of reaction centers arising in the interior of helical regions. If this were so, the reaction could not be zero order even part of the time. With calf thymus DNA, von Hippel and Wong found that their kinetic parameters were relatively insensitive to degradation of their samples by sonication down to a molecular weight of 5×10^5 (about 770 base pairs). Our observed rates, however, are extremely sensitive to the molecular weight of the preparations (Figure 1). This shows that the "end" reaction dominates. Further, there is no detectable sigmoidal character in our kinetic curves. As observed by Lazurkin and his colleagues with T2 DNA, kinetic curves representing the reaction beginning at interior sites are sigmoidal. This persists with shear degraded DNA down to a molecular weight of 2.5×10^6; by 2.2×10^5 (about 340 base pairs) the sigmoidal character has all but disappeared and the kinetics are very much influenced by the reaction at the ends (Trifonov et al., 1968).

As shown in Figure 10, our kinetic curve for higher molecular lengths, however, shows the sigmoidal characteristic similar to that observed by Trifonov et al. In this experiment, poly(A·U) were prepared from equimolar poly(A) of lengths $N = 1522$ and poly(U) of lengths as given in the figure. The dashed and solid lines were obtained by using the master equation (Chay and Stevens, 1974) for monodispersed and polydispersed cases, respectively (Chay, 1977a). Figure 11 shows the transformed plot (i.e., $\ln[D_\infty - D_t)/(D_\infty - D_0)]/t$

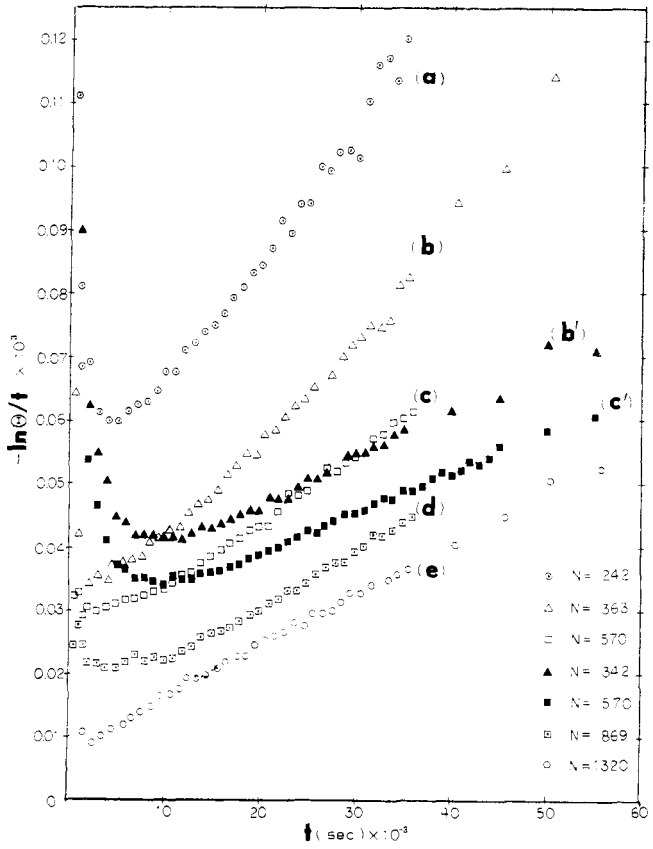


FIGURE 11: The transformed plot of Figure 10. Curves b′ and a′ were obtained from poly(A) and poly(U) of about equal lengths.

vs. t) of Figure 10. Curves b′ and c′ were obtained from the poly(A·U) prepared from the molecular lengths of poly(A) = 342 and poly(U) = 363 (curve b′) and of poly(A) = 572 and poly(U) = 570 (curve c′). By comparing the curves b and b′ (and also c and c′), we find that there must be some polydispersity in the preparation of b′ and c′.

Appendix A

The derivation leading to eq 3 is given below. According to eq 2, the following rate expressions for poly(A·U)_N and poly(A·U)_{N-1} can be written

$$\frac{d[\text{poly(A·U)}_N]}{dt} = -k_1[\text{poly(A·U)}_N] + k_2[\text{poly(A·U)}_{N-1}]$$

$$\frac{d[\text{poly(A·U)}_{N-1}]}{dt} = k_1[\text{poly(A·U)}_N] - (k_2 + k_3F)[\text{poly(A·U)}_{N-1}] + k_4[\text{poly(A·U}_f)_{N-1}]$$

where F is the concentration of formaldehyde. If we add these two rate equations and write

$$C_0(t) = [\text{poly(A·U)}_N] + [\text{poly(A·U)}_{N-1}]$$

we obtain

$$\frac{dC_0(t)}{dt} = -k_3F[\text{poly(A·U)}_{N-1}] + k_4[\text{poly(A·U}_f)_{N-1}] \quad \text{(A1)}$$

Since the species poly(A·U)_N and poly(A·U)_{N-1} are in quasiequilibrium with each other, the ratio $[\text{poly(A·U)}_N]/[\text{poly(A·U)}_{N-1}]$ should be constant for all t and equal to the helix stability parameter s. Similarly, the ratio $[\text{poly(A·U}_f)_{N-1}]/[\text{poly(A·U}_f\text{U)}_{N-2}]$ is equal to s for all t. Thus, the rapid equilibrium assumption yields

$$[\text{poly(A·U)}_{N-1}] = \frac{1}{1+s}C_0(t) \quad \text{(A2)}$$

$$[\text{poly(A·U}_f)_{N-1}] = \frac{s}{1+s}C_1(t) \quad \text{(A3)}$$

where

$$C_1(t) = [\text{poly(A·U}_f)_{N-1}] + [\text{poly(A·U}_f\text{U)}_{N-2}]$$

Thus, replacing eq A2 and A3 into eq A1, we obtain

$$\frac{dC_0(t)}{dt} = -\frac{k_3F}{1+s}C_0(t) + \frac{k_4s}{1+s}C_1(t)$$

This rate equation may be represented by the following simple kinetic scheme:

$$C_0(t) \underset{k_b}{\overset{k_f}{\rightleftharpoons}} C_1(t)$$

The rates k_f and k_b in the above equation are equal to

$$k_f = k_UK_UF/(1+s)$$

$$k_b = k_Us/(1+s)$$

where $k_U = k_4$ and $k_UK_U = k_3$. This process is repeated at the second base pair,

$$C_1(t) \underset{k_b}{\overset{k_f}{\rightleftharpoons}} C_2(t)$$

and is characterized by the same backward parameter, the forward rate parameter however, being different from k_f:

$$k_f = k_U(1 + K_UF)/(1+s) \quad \text{(A4)}$$

The factor $(1 + K_UF)$ instead of K_UF is due to the fact that the apparent reaction rate is substituted for the actual reaction rate and is an approximation which will take care of the reversibility of the imino-HCHO reaction.

This process of breaking and formalization in a sequential manner continues, each step being characterized by the same rate constants, k_f and k_b, until the final step which frees the two chains:

$$C_0(t) \underset{k_b}{\overset{k_f}{\rightleftharpoons}} C_1(t) \underset{k_b}{\overset{k_f}{\rightleftharpoons}} C_2(t) \underset{k_b}{\overset{k_f}{\rightleftharpoons}} \cdots \underset{k_b}{\overset{k_f}{\rightleftharpoons}} C_{N-2}(t) \underset{\sigma k_{bN}}{\overset{k_f}{\rightleftharpoons}} C_{N-1}(t) \quad \text{(A5)}$$

The value of k_f is unchanged for the last step; however, the reverse constant now becomes

$$k_{bN} = k\sigma s/(1 + \sigma s) \quad \text{(A6)}$$

The factor σ takes care of the gain of translational entropy from the separation of the polynucleotide chains and also accounts for the absence of a stacking interaction for the last pair. Thus, σ has a similar significance as attached to it in the discussion of equilibrium helix–coil theory in the nomenclature of Zimm and Bragg (1959).

Appendix B

Define $\theta_N(t)$ as the fraction of helical residues at time t. Then

$$\theta_N(t) = \frac{1}{N}\{N[\text{poly(A·U)}_N] + (N-1)[\text{poly(A·U)}_{N-1} + \text{poly(A·U}_f)_{N-1}] + \cdots + 1[\text{poly(A·U)}_2 + \text{poly(A·U}_f)_1]\}$$

In terms of C_i and s, the helix stability parameter, $\theta_N(t)$, is:

$$\theta_N(t) = \frac{1}{N}\left\{N\frac{s}{1+s}C_0 + (N-1)\left[\frac{1}{1+s}C_0 + \frac{s}{1+s}C_1\right] + \cdots + \left[\frac{1}{1+s}C_{N-2} + \frac{s}{1+s}C_{N-1}\right]\right\}$$

Regrouping the terms, we obtain

$$\theta_N(t) = \frac{1}{N}\left\{\sum_{i=0}^{N-2}(N-i)C_i(t) - \frac{1}{1+s}[1 - C_{N-1}(t)]\right\} \quad \text{(B1)}$$

The kinetic eq 6 can be obtained by differentiating eq B1 with respect to t:

$$N\mathrm{d}\theta_N(t)/\mathrm{d}t = \sum_{i=0}^{N-2}(N-i)\mathrm{d}C_i(t)/\mathrm{d}t + \frac{1}{1+s}\mathrm{d}C_{N-1}(t)/\mathrm{d}t \quad \text{(B2)}$$

The above equation can be evaluated using the simple kinetic laws that yield the following rate equations for process A5:

$$\mathrm{d}C_0/\mathrm{d}t = -k_{\text{fl}}C_0(t) + k_bC_i(t)$$

$$\mathrm{d}C_i/\mathrm{d}t = k_{\text{fl}}C_0(t) - (k_f + k_b)C_1(t) + k_bC_2(t)$$

$$\mathrm{d}C_i/\mathrm{d}t = k_fC_{i-1})t) - (k_f + k_b)C_i(t) + k_bC_{i+1}(t) \text{ for } 2 < i < N-3$$

$$\mathrm{d}C_{N-2}(t)/\mathrm{d}t = k_f C_{N-3}(t) - (k_f + k_b)C_{N-2}(t) + \sigma k_{bN}C_{N-1}(t)$$

$$\mathrm{d}C_{N-1}(t)/\mathrm{d}t = k_fC_{N-2}(t) - \sigma k_{bN}C_{N-1}(t) \quad \text{(B3)}$$

Introducing B3 in B2, most of the terms cancel leaving

$$\theta_N(t) = 1 - \frac{(k_f - k_b)}{N}t - \frac{(k_b - k_f + k_{\text{fl}})}{N}\int_0^t C_0(t)\mathrm{d}t + \frac{(k_f - k_b + \sigma k_{bN})}{N}\int_0^t C_{N-1}(t)\mathrm{d}t - \frac{1}{N(1+s)}\int_0^t [1 - C_{N-1}(t)]\mathrm{d}t \quad \text{(B4)}$$

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